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TITLE OF THE INVENTION

LIGHT PROMOTES REGENERATION AND FUNCTIONAL RECOVERY AFTER SPINAL CORD INJURY

Field of the Invention

The present invention generally relates to the treatment of spinal cord injury and, in particular, to the regeneration of axons within the central nervous system.

BACKGROUND OF THE INVENTION

Spinal cord injury (SCI) is a serious clinical problem with approximately eight thousands new cases every year. SCI occurs when a traumatic event results in damage to cells within the spinal cord or severs the nerve tracts that relay signals up and down the spinal cord. The most common types of SCI include contusion and compression. Other types of injuries include lacerations, and central cord syndrome (specific damage to the corticospinal tracts of the cervical region of the spinal cord). Severe SCI often causes paralysis and loss of sensation and reflex function below the point of injury, including autonomic activity such as breathing and other activities such as bowel and bladder control. SCI patients are also prone to develop secondary medical problems, such as bladder infections, lung infections, and bed sores.

Damage to spinal cord is often permanent due to the inability of axons within the central nervous system to regenerated following an injury. While recent advances in emergency care and rehabilitation allow many SCI patients to survive, methods for reducing the extent of injury and for restoring function are still

limited. Immediate treatment for acute SCI includes techniques to relieve cord compression, prompt (within 8 hours of the injury) drug therapy with corticosteroids such as methylprednisolone to minimize cell damage, and stabilization of the vertebrae of the spine to prevent further injury.

Light therapy (LT), also known as photo-biomodulation or low power laser irradiation (LPLI), is a non-invasive treatment which evokes biological effects via the absorption of light. LPLI has been shown to increase neuronal survival and regeneration in the peripheral nervous system [Anders, et al., *Surg. Med*, 13:72-82 (1993), Snyder, et al., *Surg. Med*, 31:216-222 (2002)]. Investigation has shown that LT, through the absorption of light by a cellular photoreceptor, rather than heating of the cell [Anders, et al., *Surg. Med.* 13:72-82, (1993), and Mochizuki-Oda, et al., *Neurosci. Lett.* 323:207-210 (2002)], can increase or decrease ATP, DNA, RNA and protein synthesis, depending on the treatment parameters applied [Saperia, et al., *Biochem. Biophys. Res. Commun.* 138:1123-1128 (1986); Greco, et al., *Biochem. Biophys. Res. Commun.* 163:1428-1434 (1989); Lam, et al., *Lasers Life Sci.* 1:61-77 (1986); Funk, et al., *J. Photochem. Photobiol. B:BBiol.* 16:347-355 (1992); Mochizuki-Oda, et al., *Supra* (2002)].

LT research, however, is not extensive within the area of CNS injury and no study to date has assessed the ability of light to regenerate specific tracts within the spinal cord or determined the recovery of specific locomotor functions.

In sum, although progress has been made in recent years in the treatment of SCI, there exists a need to develop new treatment for SCI, to improve the efficiency of the existing method such as LT, and to develop methods to better evaluate the effectiveness of the new treatment modalities.

SUMMARY OF THE INVENTION

The present invention relates generally to the treatment of SCI by stimulating axon regeneration within the central nerve system. One aspect of the present invention provides methods of treating SCI with low power laser irradiation (LPLI). Another aspect of the present invention provides methods of treating SCI by modulating a gene activity to stimulate axon regeneration. In this regard, the present invention also provides compositions that modulate genes expression relating to the neuron- regeneration after SCI. Another aspect of the present invention provides methods for evaluating the effectiveness of a treatment for SCI..

DESCRIPTION OF THE FIGURES

Figure 1. a) Photograph of spectrophotometric analysis experimental set-up. The smart fiber (arrow) is inserted below the skin of the rat, the light source (arrowhead) is positioned above the skin for transcutaneous application of light. b) Graphical representation of transmission (in arbitrary units) through each layer of tissue, depending on wavelength (nm). Layer 1 = skin, 2 = loose connective tissue, 3 = dense connective tissue, 4 = muscle, 5 = vertebral column and spinal cord. The graph demonstrates that wavelengths in the 770 - 810 nm range had the greatest transmission, or penetration, through all levels. c) Human spinal section transmission / scatter measurements. Sampling fiber diameter is 1 mm.

Figure 2 are the photomicrographs of mini-ruby labeled axons and related quantitation. a) Photomicrograph of white matter 4 mm caudal to lesion site in control rat. b) Photomicrograph of white matter 4 mm caudal to lesion in light treated rat. Note that mini-ruby labeled axons, indicated with arrows, are found at this distance only in the light treated group. Bar = 43μm. Comparisons of average axon number/animal (c) and average distance caudal to the lesion (d) are shown.

*p<0.01; **p<0.001 using one way ANOVA. e) Number of axons counted in control and light treated groups per mm caudal to the lesion. Bars represent mean +/- SEM.

Figure 3. Photomicrographs of single and double labeled neurons at 10 weeks post-injury. a) Numbers of HM labeled neurons (arrowheads) in the motor cortex, b) fast blue labeled neurons in the injection site at L3, and c) fast blue labeled neurons in the motor cortex were similar in both groups. d) Graphical representation of comparison of double labeled neurons between light treated and control groups. *p<0.05 using Mann Whitney U. Bars represent mean percentage of counted neurons +/- SEM. e - g) Double labeled neurons were found only in motor cortex of light treated rats. Arrows indicate double labeled neurons, identifiable by green punctate label in blue cytoplasm, which is consistent with labeling pattern previously described. [Pyner et al., *Neuroscience* 100:549-556 (2000)]. Bar = 67μm (a - e); 34μm (f - g).

Figure 4. Angle of rotation (a), footfalls (b) and ladder beam crossing time (c) measurements are presented for pre-surgical, 1 week and 9 weeks postsurgical time points. Significant improvement was found only in the light treated group. Graph bars are mean percentage of pre-surgical measurements +/SEM. *p<0.05 using repeated measures ANOVA with Newman Keuls post-test between time points. **p<0.05 using one way ANOVA with Tukey post-test between control and light treatment group at 9 week time point. d) Footprints from pre-surgical and 9 weeks post-surgical analysis. Notice the increased angle of rotation and smearing at 9 weeks in the control group. In the light treated group, the angle is similar to pre-surgical values and there is no smearing of the footprint.

Figure 5. Gross observation of longitudinal H&E stained sections of the thoracic spinal cord. H&E representative sections for control (a, c) and light treated (b, d) groups. Increases in cavity size were observed by 4 dpi in both groups (a, b)

compared to 48 hours post-injury. By 14 dpi, spread of cavitation away from the center of the lesion (*) was greater in the control group (c) than the light treated group (d), particularly in the rostral direction. Cavitation was more prominent in the white matter (w) than in the gray matter (g). All tissue is oriented in the same rostral/caudal direction. Bar = 250 mm.

Figure 6. Immunohistochemistry of neutrophils, macrophages and activated microglia. Immunohistochemistry for neutrophils (arrows; a, b) was found in 4 dpi tissue from both control (a) and light treated (b) groups. Quantitation of immunolabeling for neutrophils is shown in (c). Immunolabeling for macrophages/activated microglia is demonstrated in (d-h). Panel (d) is a control section from 14 dpi, demonstrating cells accumulated in and around the lesion site (*). e) Light treated tissue at 14 dpi. f) Quantitation of immunolabeling for macrophage/activated microglia. g) 16 dpi control tissue. h) 16 dpi light treated tissue. i) Negative control tissue. *$p<0.001$ between the control and light treated groups; n=5/group; ANOVA followed by Tukey test. Graph bars represent mean +/- SEM. Bar = 95 mm.

Figure 7. Immunohistochemistry of astrocytes.

Figure 8. Immunohistochemistry of T and B lymphocytes and Schwann cells. Images of 14 dpi tissue from control and light treated tissue are demonstrated in this figure. Immunolabeling for T lymphocytes (arrows a, b) was restricted to the lesion site (*). a) Control tissue and b) light treated tissue at 14 dpi. c) Quantitation of T lymphocyte immunolabeling. *$p<0.05$ between control and light treated tissue. d) B lymphocyte immunolabeling (arrows) in control tissue. h) Quantitation of B lymphocytes immunolabeling. Schwann cell immunolabeling (arrows) near the lesion site (*) in control (f) and light treated (g) tissue. h) Quantitation of Schwann cell immunolabeling. Graph bars represent mean +/- SEM (n = 5/group; ANOVA followed by Tukey test). Bar = 96 mm.

Figure 9. GAPDH comparison. Internal control (GAPDH) expression revealed no significant difference between the control and LT groups (data from 6 hours and 4 days post-injury collapsed into one group; no significant difference seen for individual time points: data not shown). $p>0.05$ (Students t-test); bars represent mean +/- SEM.

Figure 10. Pro-inflammatory cytokine expression. Four pro-inflammatory cytokines were semi-quantitated in this study. All samples were from the site of spinal cord injury at 6 hours (H) and 4 days (D) post-injury. A) IL6 expression analysis revealed significant inhibition of IL6 mRNA production by LT at 6 hours post-injury, but no significant difference between groups at 4 days post-injury. B) Ethidium bromide-DNA complex fluorescence for IL6 from the control and LT groups, as well as their corresponding GAPDH band, at 6 hours post-injury was digitally photographed. C) IL 1b expression. D) TNFα expression. E) GM-CSF expression. *$p<0001$ for comparison between control and LT group at individual time point (ANOVA followed by Tukey post-test), bars represent ratio of gene of interest to internal control mean +/- SEM.

Figure 11. Chemokine expression. Two chemokines were assessed in this study. All samples were from the site of spinal cord injury at 6 hours (H) and 4 days (D) post-injury. A) MCP-1 expression at 6 hours and 4 days after SCI. B) Representative gel bands for MCP-1 from the control and LT groups, as well as their corresponding GAPDH band. C) MIP1a expression at 6 hours and 4 days after SCI. *$p<0.01$ for comparison between control and LT group at individual time point (ANOVA followed by Tukey post-test), bars represent ratio of gene of interest to internal control mean +/- SEM.

Figure 12. INOS, ICAM and TGFβ expression. Expression of iNOS, ICAM and TGFβ were assessed at 6 hours (H) and 4 days (D) post-injury. A) iNOS expression at 6 hours and 4 days post-injury. B) ICAM expression. C)

TGFβ expression. *p<0.01 for comparison between control and LT group at individual time point (ANOVA followed by Tukey post-test), bars represent ratio of gene of interest to internal control mean +/- SEM.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention relates to treatment of SCI using LPLI. It is yet unclear exactly what happens to light as it propagates through human tissues, particularly, when more then one tissue type is involved (e.g., skin, muscle, bone, etc.). The analytical solutions of the problem are quite complex and mostly intractable, or, when simplified, inaccurate. The numerical solutions are oversimplifications, or require unavailable/unreliable data, and once again give inaccurate solutions. Early measurements of light propagation in animal tissues suggested that "more light gets through to the deep tissues" than one would expect from the simple analytical solutions or the numerical solutions using ex-vivo measurements of single tissues i.e. using data from existing literature to analyze the propagation of light through complex/multiple tissue type "samples" would produced overly pessimistic results. The measurements taking in cadavers seem to confirm these earlier suspicions.

While the expectation/knowledge that more light gets through than predicted makes non-invasive, transcutaneous delivery of light in the treatment of deep tissue possible, relatively large amounts of energy are still needed at the surface of the skin. Most of the energy at the skin will be absorbed by the tissue between the skin and the target tissue; such absorption will increase the tissue's temperature at a rate proportional to the power density, i.e., the number of photons per unit area per unit time.

The LPLI therapy of the present invention is designed to minimize the required power density at the skin while accounting for the tissue scattering to deliver appropriate "treatment doses" to the target tissue. In one embodiment,

laser light with a wave length of 750-850 nm, preferably about 770-820nm, most preferably about 810 nm, and an output power of 50mW-50W, preferably about 100mW -6W, most prefer about 125mW-5.5W is applied transcutaneously to a mammal at the site of acute injury to the corticospinal tract (CST) for 7-21 consecutive days and preferably about 14 days. Preferably, the treatment begins immediately after the injury. As is well understood by one skilled in the art, the light density, output, total daily dosage, and the length of the treatment period may vary depending on the form, severity, and site of the particular SCI.

Figure 1 shows the light transmission through various kinds of tissues between the light source and spinal cord. The peak penetration through all tissue layers (i.e., skin, loose connective tissue, dense connective tissue, muscle, and vertebral column) to the spinal cord was found between the 770 nm and 810 nm wavelengths (Figure 1b) . Figure 1c shows the transmitted light intensity distribution in the human spinal section. The transmission measurements is described in Table 1.

In another embodiment, injured rat spinal cord is transcutaneously irradiated at the lesion site for a total of 14 days with an 810 nm diode laser (Thor International, UK) with 150 mW output through a delivery fiber optic (2,997 seconds treatment time/day). The daily dosage was 1589 J/cm^2 (irradiance = 0.53 W/cm^2, 450 J).

As shown in Figure 2 anterograde mini-ruby analysis reveals that the average number of mini-ruby labeled axons in the light treated group was significantly greater than that of the control group ($p<0.000\ 1$, one way ANOVA with Tukey post-test; Figure 2a), with an average of 199.2 +/- 12.46 labeled axons caudal to the lesion. The mini-ruby labeled axons in the light treated group extended an average of 8.7 +/- 0.75 mm caudal to the lesion, significantly longer than the control group ($p<0.01$, one way ANOVA with Tukey post-test; Figure 2b).

The maximum distance traveled over 5 weeks in the light treated group was 14 mm caudal to the lesion, which was reached by 8% of the counted axons (Figure 2e).

In another preferred embodiment, the effectiveness of the LPLI treatment is confirmed by a double-label, retrograde tracing analysis. At the time of CST lesion, transected neurons are labeled by an anterograde tracer, hydroxystilbamidine methanesulfonate (HM), inserted into the lesion. Ten weeks after CST lesion, axons terminating at vertebral level L3, approximately 24 mm caudal to the initial lesion, are labeled by injection of fast blue into the ventral horn. Numbers of single (HM or fast blue) and double (neurons with axons that are transected and regrew to L3) labeled neurons in the motor cortex are assessed using unbiased stereology.

As shown in Figure 3, in one embodiment, double labeled neurons, with both HM and fast blue labeling, are found only in the light treated group (Figure 3d, e, f) and the percentage of these neurons represented a statistically significant increase in comparison to the control group (Figure 3d, $p<0.05$, Mann-Whitney U Test). This increase in double labeling indicates that only CST axons in the light treated group regrew and terminated in the gray matter of vertebral level L3 after transaction.

In yet another preferred embodiment, the axonal regeneration and reinnervation are evaluated by two functional tests, the ladder/grid walking test and footprint analysis, preformed prior to and after CST lesion. The measurements taken include footfalls (failure of handpass to grasp ladder rungs and falling below the plane of the ladder), time to cross the ladder, base of support, stride length, and angle of rotation. As shown in Figure 4, in one embodiment, one week after CST lesion, experimental rats had significant impairments in angle of rotation ($p<0.05$, Figure 4a, repeated measures ANOVA with Newman-Keuls post-test) and footfalls ($p<0.05$, Figure 4b) in comparison to pre-surgical measurements in both control

and light treated groups. An increase in ladder cross time was also observed in both groups at this time point (Figure 4c).

At 9 weeks post-injury, animals in the light treated group had no significant difference ($p>0.05$; Figure 4a, c) in angle of rotation (Figure 4d) and ladder beam cross time in comparison to the pre-surgical measurements, demonstrating a recovery of these functions. Comparison of ladder beam cross time and angle of rotation measurements in light treated and control groups also revealed a significant improvement in the light treated group ($p<0.05$, one way ANOVA with Tukey post-test; Figure 4a, c). Measurements for rats that received CST lesions but were not light treated remained at the 1 week post-surgery levels at this time point, significantly greater than pre-surgical measurements ($p<0.05$).

These measurements suggest that LT promotes significant improvement in specific CST controlled functions after lesioning, and demonstrate particular improvement in functions mediated by innervation from the L1-L3 vertebral level.

In yet another embodiment, the effectiveness of effectiveness of the LPLI treatment is evaluated by cell invasion and activation. In one embodiment, the dorsal halves of spinal cords are lesioned in adult rats and transcutaneously irradiated for a maximum of 14 days, beginning immediately after surgery, using a 150 mW 810 mn laser (1589 J/cm^2) . The response of macrophages/activated microglia, neutrophils, T and B lymphocytes, astrocytes and Schwann cells to SCI and LPLI is quantified by immunohistochemistry at 2, 4, 14 and 16 days and 5 weeks post-injury. As shown in Figures 5-8, LPLI suppress invasion/activation of macrophages and microglia as well as T lymphocytes. The activation of astrocytes is delayed two days by LT, with a peak in activation occurring at 4 days post-injury. A similar trend is found in the migration and activation of B lymphocytes.

and neutrophils, in which decreases are found in the light treated group, although these are not statistically significant. LPLI has no effect on Schwann cell migration into the spinal cord. These results indicate that light alters the spinal cord environment and the immune response following SCI.

Another aspect of the present invention relates to cytokine and chemokine genes as markers and therapeutical targets/agents of SCI. Cytokines and chemokines are integral in the inflammatory response of tissue to injury. Following SCI, cytokines and chemokines are upregulated and play an important role in cellular invasion/activation and secondary damage. LPLI has significant effects on the inflammatory response of cells in vitro and in various in vivo injury models. The impact of LPLI on cytokine and chemokine gene expression after SCI are evaluated. Specifically, reverse transcriptase - polymerase chain reaction is used to detect the expression of several genes, including the pro-inflammatory cytokines interleukin (IL) 1β , tumor necrosis factor α, IL6 and granulocyte-macrophage colony-stimulating factor, the chemokines macrophage inflammatory protein 1 and monocyte chemoattractant protein (MCP-1), as well as inducible nitric oxide synthase (iNOS), intercellular adhesion molecule and transforming growth factor β. All genes studied are expressed after SCI in both the LPLI and control groups, however, expression of IL6, MCP-1 and iNOS is significantly suppressed in the LPLI group. In one experiment, IL6 expression is 171 fold greater in the control group than the LPLI group at 6 hours post-injury ($p<0.001$). MCP-1 and iNOS are also suppressed at 6 hours post-injury by LPLI, with 3 and 5 fold decreases ($p<0.01$), respectively. These genes are hereby designated as SCI-related genes (SRGs).

Therefore, without limitation as to mechanism, the invention is based in part on the principle that modulation of the expression of an SRG may ameliorate SCI when the SRG is expressed at levels similar or substantially similar to that in LPLI treated animals. Similarly, modulation of an activity of an SRG product (i.e. a protein encoded by an SRG or a polynucleotide transcribed from an SRG) may ameliorate SCI when the activity of the SRG product is at levels similar or substantially similar to that in LPLI treated animals.

In one aspect, the invention provides SRGs whose level of expression, which signifies their quantity or activity, is correlated with the presence of SCI. In certain preferred embodiments, the invention is performed by detecting the presence of an SRG product. In another aspect of the invention, the expression levels of SRGs are determined in a particular subject sample for which either diagnosis or prognosis information is desired. The level of expression of a number of SRGs simultaneously provides an expression profile, which is essentially a "fingerprint" of the presence or activity of an SRG or plurality of SRGs that is unique to the state of the cell or tissue. In certain embodiments, comparison of relative levels of expression is indicative of the severity of SCI, and as such permits for diagnostic and prognostic analysis. Moreover, by comparing relative expression profiles of SRGs from tissue samples taken at different points in time, *e.g.*, pre- and post-therapy and/or at different time points within a course of therapy, information regarding which genes are important in each of these stages is obtained. The identification of genes that are differentially expressed in SCI versus normal tissue, as well as differentially expressed genes after SCI, allows the use of this invention in a number of ways. For example, comparison of expression

profiles of SRGs at different stages of the SCI provides a method for long-term prognosis. In another example mentioned above, the evaluation of a particular treatment regime may be evaluated, including whether a particular drug will act to improve the long-term prognosis in a particular patient.

The discovery of these differential expression patterns for individual or panels of SRGs allows for screening of test compounds with the goal of modulating a particular expression pattern. For example, screening can be done for compounds that will convert an expression profile for a poor prognosis to one for a better prognosis. In certain embodiments, this may be done by making biochips comprising sets of the significant SRGs, which can then be used in these screens. These methods can also be done on the protein level; that is protein expression levels of the SRGs can be evaluated for diagnostic and prognostic purposes or to screen test compounds. For example, in relation to these embodiments, significant SRGs may comprise SRGs which are determined to have modulated activity or expression in response to a therapy regime. Alternatively, the modulation of the activity or expression of an SRG may be correlated with the diagnosis or prognosis of SCI. In addition, the SRGs can be administered for gene therapy purposes, including the administration of antisense nucleic acids, or proteins (including dominant mutants of SRG encoded proteins, antibodies to SRG encoded proteins and other modulators of SRG encoded proteins) administered as therapeutic drugs.

In another embodiment of the invention, a product of an SRG, either in the form of a polynucleotide or a polypeptide, can be used as a therapeutic compound of the invention. In yet other embodiments, a modulator of SRG expression or the

activity of n SRG product may be used as a therapeutic compound of the invention, or may be used in combination with one or more other therapeutic compositions or methods of the invention. Administration of such a therapeutic may suppress bioactivity of an SRG product, and therefore may be used to ameliorate SCI.

Examples

Example 1. Spectrophotometric measurement

An incoherent broad band white light was directed at the surface of the skin in the low thoracic region of adult Sprague Dawley rat. Rats were anesthetized with sodium pentobarbital (50 mg/Kg, I.P.) prior to all measurements. A smart, tissue-activated optical fiber probe [Ilev, I et al., *Proc Spie* 4616:220-228 (2002)] was inserted sequentially into the skin, sub-cutaneous connective tissue layer, deep connective tissue layer, muscle and the spinal cord within the vertebral column. At each of these layers, a transmission spectrum in the range of 500 - 1200 nm was collected while white light was applied to the skin surface.

Example 2. Corticospinal tract lesion

Thirty adult female Sprague Dawley rats were used in this study. For all surgical techniques, rats were anesthetized with sodium pentobarbital (50mg/Kg, I.P.). Dorsal hemisection was performed by an investigator blinded to group assignment. The ninth thoracic vertebra was identified and a laminectomy was performed to expose the spinal cord. A suture was passed beneath the dorsal funiculus. Iridectomy scissors were used to carefully incise this isolated portion of the spinal cord, transecting the CST. Inspection of the lesion and visualization of the central gray commissure verified that the CST had been completely transected. After the hemisection was completed, the exposed spinal cord was covered with

gelfoam (Pharmacia, Upjohn; Kalamazoo, MI), and the overlying muscles and skin were sutured. During the recovery period, urinary bladders were manually expressed until spontaneous voiding returned approximately 1-2 days post-injury.

Example 3. Retrograde labeling

At the time of CST lesion, gelfoam soaked in hydroxystilbamidine methanesulfonate (HM; 3% in 0.9% saline; Molecular Probes, Eugene, OR) was inserted into the lesion site of 20 rats. Ten weeks after the surgery, a laminectomy was performed at vertebral level L3, approximately 24 mm caudal to the original lesion site, and 1 μl of a 2% fast blue solution (in PBS, Sigma, St. Louis, MO) was bilaterally injected (0.5 μl into each side) into the spinal cord at a depth of 1.3 mm.

Example 4. Anterograde labeling

Five weeks after CST lesion, 10% tetramethylrhodamine biotinylated dextran (mini-ruby, Molecular Probes) was injected into the motor cortex of one group of 10 rats using stereotaxic coordinates (from bregma, -0.11 AP and ±1.60 ML; -1.33 AP and ±1.50 ML; -2.85 AP and ±1.40 ML; depth = 1.0 - 1.2 mm). 2 μl of mini-ruby was injected into each of the 6 sites, for a total injection volume of 12 μl.

Example 5. Tissue analysis for labeling detection

Eight days after the injections of mini-ruby or fast blue, rats were perfused with 4% paraformaldehyde. Brains and spinal cords were carefully dissected, post-fixed for 24 hours and cryoprotected in 30% sucrose for 24 hours prior to sectioning of the tissue at a thickness of 20 μm. Sections for counting mini-ruby labeled axons from the lesion site to 16 mm caudal to the lesion were collected and

mounted at a ratio of 1/6. For neuronal counting, cortical sections were collected and mounted at a ratio of 1/8. The fractionator method of unbiased stereology was used to count HM and/or fast blue labeled neurons in the motor cortex (2.6 mm from midline to lateral edge of brain per hemisphere). The percentage of neurons that regenerated an axon was calculated according to:

$$\frac{\text{Double labeled neuron}}{\text{Fast Blue + HM + Double Labeled neurons}} \times 100$$

Cortical and spinal cord injection sites were studied prior to counting to ensure labeling efficacy; only those with adequate injections, without leakage of the tracer significant distances away from the injection site, and with adequate uptake into the intended neurons, were included in the final analysis.

Example 6. Light treatment

Beginning immediately after surgery, half of the rats (randomly assigned; n=15/group), were transcutaneously irradiated at the lesion site for a total of 14 consecutive days with an 810 nm diode laser (Thor International, UK; 150 mW output through a delivery fiber optic, 2,997 seconds treatment time/day). Dosage was 1589 J/cm^2 per day (irradiance = 0.53 W/cm^2, 450 J).

Example 7. Functional testing

One week prior, and 1 and 9 weeks after dorsal hemisection surgery, the same rats undergoing retrograde labeling were trained and then tested on two functional tests. One test required rats to walk across a ladder beam (Columbus Instruments, Columbus, OH) that recorded the length of time required to cross the beam as well as the number of footfalls. This test was videotaped for confirmation.

Rats also underwent footprint analysis: handpass were dipped in ink and the rats walked across sheets of white paper. Base of support, stride length and angle of rotation were analyzed as described previously [Kunkel-Bagden, E et al., *Exp Neurol* 119:153-164 (1993); Hamada Y, et al., *J Neurochem* 66:1525-1531 (1996)].

Example 8. Statistical analysis

Functional test data are presented as mean percentage of baseline scores recorded one week prior to surgery +/- SEM. Neuronal counts are presented as mean percentage of total neuronal number counted +/- SEM. Axonal counts are presented as mean +/- SEM. Functional data were analyzed using Repeated Measures ANOVA with Newman-Keuls post-test to assess changes over time or one-way ANOVA with Tukey post-test to assess differences between groups at individual time points. Axonal count data were analyzed using One Way ANOVA, with Tukey posttest. Neuronal count data were analyzed using Mann-Whitney U analysis.

Example 9. Spectrophotometric analysis LPLI

A series of experiments involving *in vivo* spectrophotometric analysis were performed to assess whether transcutaneous application of 810 nm laser diode emission with an output power of 150 mW was able to penetrate to the depth of the spinal cord (Figure 1a). Peak penetration through all tissue layers to the spinal cord was found between the 770 nm and 810 mn wavelengths (Figure lb). Six percent, or approximately 9 mW, of the initial power output penetrates to the spinal cord. These data show that 810 nm light, with an adequate amount of energy, reaches the spinal cord.

Example 10. Anterograde tracer analysis of axon regeneration after LPLI treatment of SCI

To determine if application of 810 nm light to the spinal cord increased axonal growth, Ln anterograde tracer, mini-ruby (Molecular Probes, Eugene, OR), was injected 5 weeks after CST lesion. Analysis revealed that all mini-ruby labeled axons were found in the white matter, in the region of the spinal cord normally occupied by the CST (Figure 2a, b). There were few (30.72 +/- 16.9 axons per animal) mini-ruby labeled axons caudal to the lesion in the control group (Figure 2c). These labeled axons extended. an average distance of 2.9 +/- 0.84 mm caudal to the lesion (Figure 2d), with a maximal distance of 7 mm reached by 17% of counted axons (Figure 2e), which is comparable to spontaneous post-lesional sprouting previously reported 6 . The average number of mini-ruby labeled axons in the light treated group was significantly greater than that of the control group ($p<0.000\ 1$, one way ANOVA with Tukey post-test; Figure 2a), with an average of 199.2 +/- 12.46 labeled axons caudal to the lesion. The mini-ruby labeled axons in the light treated group extended an average of 8.7 +/- 0.75 mm caudal to the lesion, significantly longer than the control group ($p<0.01$, one way ANOVA with Tukey post-test; Figure 2b). The maximum distance traveled over 5 weeks in the light treated group was 14 mm caudal to the lesion, which was reached by 8% of the counted axons (Figure 2e).

Example 11. Double-label analysis of axon regeneration after LPLI treatment of SCI

Anterograde analysis does not definitively determine whether transected axons have regrown past the lesion, as spared axons may also be counted caudal to the lesion. Therefore, to determine if LPLI promotes regeneration of transected axons, a double label, retrograde tracing analysis was performed. Based on the mini-ruby data, axons in the light treated group grew at a rate of 0.25 - 0.4 mm per day. Using this data, we calculated that axons would require at least 10 weeks in order to reach the mid-lumbar region and innervate motor neurons responsible for

lower limb function. At the time of CST lesion, transected neurons were labeled by an anterograde tracer, hydroxystilbamidine methanesulfonate (HM), inserted into the lesion. Ten weeks after CST lesion, axons terminating at vertebral level L3, approximately 24 mm caudal to the initial lesion, were labeled by injection of fast blue into the ventral horn. Numbers of single (HM or fast blue) and double (neurons with axons that were transected and regrew to L3) labeled neurons in the motor cortex were assessed using unbiased stereology.

Analysis of single labeled neuron number revealed no significant difference ($p<0.05$) between control and light treated groups, suggesting no difference in labeling efficacy between groups (Figure 3a, b, c). The average number of HM labeled neuronsis 8,860 +/- 3408 in the control group and 13,270 +/- 3236 in the light treated group, which is comparable to the number of CST axons reported in the lower thoracic region of the spinal cord [Brosamle, C. et al., *J Comp Neurol* 386:293-303 (1997); Hicks, P. et al., *Exp Neurol* 56: 410-420 (1977)]. The average number of fast blue labeled neurons is 128.9 +/108.6 in the control group and 130.9 +/- 119.8 in the light treated group, which is comparable to the number of neurons found in the motor cortex after injection of a retrograde tracer into the ventral, uncrossed portion of the CST at vertebral level L4 [Brosamle, C. et al., *J Comp Neurol* 386:293-303 (1997)]. Fast blue has been shown to spread approximately 2 mm from its injection site [Kalderon, N. et al., *Proc Nat/ Acad Sci USA* 93:11179-11184 (1996)], therefore neurons in laminae III - VI, where ventral CST axons terminate, may have taken up the tracer. Since crossed and uncrossed axons of the CST originate from the same area of the motor cortex [Brosamle, C. et al., *supra* (1997)], it is likely that these fast blue labeled neurons are from the ventral, uncrossed CST that was not lesioned in the surgical procedure. The uncrossed CST axons, however, do not play a significant role in motor function in the rat [Whishaw, Q et al., *Behav Brain Res* 134:323-36

(2002)].

Double labeled neurons, with both HM and fast blue labeling, were found only in the light treated group (Figure 3d, e, f) and the percentage of these neurons represented a statistically significant increase in comparison to the control group (Figure 3d, $p<0.05$, Mann-Whitney U Test). This increase in double labeling indicates that only CST axons in the light treated group regrew and terminated in the gray matter of vertebral level L3 after transaction.

This study revealed that double labeled neurons accounted for approximately 30% of the number of mini-ruby labeled axons observed at 5 weeks post-lesion in the light treated group. As double labeling represents neurons with axons that terminated in the L3 area, it is understandable that the total number of regenerating axons found at the T12 level at 5 weeks post-injury would be greater.

Example 12. Functional evaluation of axon regeneration after LPLI treatment of SCI

Complete lesion of the dorsal portion of the adult rat CST at vertebral level T9 results in loss of several CST controlled functions [Whishaw, Q et al., *Behav Brain Res* 134:323-36 (2002); Kunkel-Bagden, E et al., *Exp Neurol* 119:153-64 (1993)]. To determine if the axonal regeneration and reinnervation resulted in functional improvement, performance of rats in two functional tests, the ladder/grid walking test and footprint analysis, was assessed prior to and after CST lesion. Five measurements were taken, including footfalls (failure of hindpaws to grasp ladder rungs and falling below the plane of the ladder), time to cross the ladder, base of support, stride length, and angle of rotation. Data are presented as mean percentage of pre-surgical measurement, to control for variations among animals.

One week after CST lesion, rats had significant impairments in angle of rotation ($p<0.05$, Figure 4a, repeated measures ANOVA with Newman-Keuls

post-test) and footfalls ($p<0.05$, Figure 4b) in comparison to pre-surgical measurements in both control and light treated groups. An increase in ladder cross time was also observed in both groups at this time point (Figure 4c).

At 9 weeks post-injury, rats underwent these functional tests again. At this time point, animals in the light treated group had no significant difference ($p>0.05$; Figure 4a, c) in angle of rotation (Figure 4d) and ladder beam cross time in comparison to the pre-surgical measurements, demonstrating a recovery of these functions. Comparison of ladder beam cross time and angle of rotation measurements in light treated and control groups also revealed a significant improvement in the light treated group ($p<0.05$, one way ANOVA with Tukey post-test; Figure 4a, c). Measurements for rats that received CST lesions but were not light treated remained at the 1 week post-surgery levels at this time point, significantly greater than pre-surgical measurements ($p<0.05$).

Angle of rotation and ladder cross time are both associated with CST function and are significantly lengthened by CST lesion [Kunkel-Bagden, E et al., *Exp Neurol* 119:153-64 (1993); Metz, A et al., *J Neurosci Methods* 115:169-79 (2002)]. Ladder crossing time is positively correlated with hindlimb errors in step placement [Metz, A et al., *J Neurosci Methods* 115:169-79 (2002)]. We found a significant increase in footfalls in both control and light treated animals post-surgery ($p<0.05$, Figure 4b), but there was no significant difference between these two groups. However, analysis of errors in ladder crossing, including correct placement of hindpaws on ladder rungs and grasping of ladder rungs, was not assessed in this study and may have been modified by light treatment, leading to the observed improvement in crossing time.

No significant change was found in stride length or in base of support in

either group at any time point after CST lesion ($p>0.05$, data not shown). Previous studies have shown that CST lesion in adult rats does not necessarily impair these functions, as this lesion does not affect the rubrospinal or propriospinal tracts, which play a greater role in these functions than the CST [Kunkel-Bagden, E et al., *Exp Neurol* 116: 40-51 (1992); Hamers, P et al., *J. Neurotrauma* 18:187-201 (2001)].

These data suggest that LT promotes significant improvement in specific CST controlled functions after lesioning. This study demonstrated particular improvement in functions mediated by innervation from the L1-L3 vertebral level. Similar results have been found with other treatment modalities, such as transplantation of fetal tissue [Kunkel-Bagden, E et al., *Exp Neurol* 116: 40-51 (1992); Kunkel-Bagden, E. et al.,, *Exp Brain Res* 81:25-34 (1990)].

Example 13. Dorsal Hemisection of Spinal Cord

Twenty adult female Sprague-Dawley rats (200 - 300g, Taconic Farms, Germantown, NY) were used in this study under an approved Uniformed Services University IACUC protocol. Food and water were provided ad libitum and the rats were exposed to 12-hour cycles of light and dark periods.

Rats were randomly assigned to two groups (LT group, n= 10; control group, n = 10). Investigators were blinded to the group assignment prior to dorsal hemisection surgery. Animals were anesthetized with sodium pentobarbital (50mg/Kg, I.P.) and placed on an isothermal heating pad warmed to 37°C. The ninth thoracic vertebra was identified and a laminectomy was performed to expose the spinal cord between T8 and T10. The dorsal funiculus was isolated by passing a suture thread through the spinal cord. Iridectomy scissors were used to carefully incise this isolated portion of the spinal cord, thereby transecting the corticospinal

tract. Inspection of the lesion and visualization of the central gray commissure verified that the corticospinal tract had been completely transected.

After the dorsal hemisection was completed, the exposed spinal cord was covered with gelfoam (Pharmacia, Upjohn; Kalamazoo, MI), and the overlying muscles and skin were sutured. During the recovery period, bladders were manually expressed until spontaneous voiding returned at approximately 1-2 days post-injury.

Example 14. Gross observation of longitudinal H&E stained section of the thoracic spinal cord

Assessment of temporal invasion/activation of the various cell types of interest was investigated within the lesion site and the surrounding tissue. Gross observation of longitudinal H&E stained sections of the thoracic spinal cord revealed a cavity at the location of the initial injury (Fig 5). Between 48 hours and 4 dpi the size of this cavity increased (Fig 5a, b) and expanded longitudinally from 4 to 16 dpi (Fig 5a – d). This expansion extended a greater distance (1 – 2 mm) in the rostral direction than the caudal direction (0 – 0.5 mm). The cavitation spread was found primarily in the white matter of the spinal cord. The cavitation, both in the initial injury site and rostral/caudal to it, appeared to be decreased at the 5 weeks post-injury time point (data not shown). No observable difference in cavity size was seen between the control and light treated groups, however there appeared to be a decrease in longitudinal spread of the rostral cavitation in the 14 dpi light treated tissue (Fig 5d).

Due to the clustering behavior of cells within and surrounding the lesion following SC. and the inability to discern individual cell nuclei, assessment of numbers of individual cells was not possible. Therefore, measurement of tissue

area occupied by immuno-positive label within a defined target space was used to assess cell invasion/activation. As an increase in immunolabeling does not necessarily reflect an increase in cell number, this measurement is a method of quantifying the magnitude of a cellular response, both in terms of cell invasion and activation. The current work does not attempt to distinguish between these two cellular response parameters.

Example 15. Analysis of cell invasion and activation

Spinal cord tissue from rats was collected at 48 hours, 4, 14, and 16 days and 5 weeks post-injury. At each time point, 5 rats per treatment group were deeply anesthetized with 10% chloral hydrate (1 ml/100g, I.P.) and euthanized via intracardiac perfusion with 4% paraformaldehyde. The thoracic spinal cord at the lesion site and 3 mm rostral and 5 mm caudal to the lesion site was dissected, post-fixed for 24 hours in 4% paraformaldehyde, and cryoprotected for 24 hours in 3 0% sucrose. The 10 mm spinal cord segments were sectioned longitudinally on a freezing microtome at 20 μm, from the dorsal aspect of the spinal cord through the level of the gray commissure. Sections were serially mounted onto 10 slides, with 3 sections per slide. One slide from each rat was processed for histological analysis using an H&E stain and one slide/rat was processed for each cell type under investigation. Immunolabeling was repeated for each animal to ensure labeling efficacy. Negative controls, in which primary antibody was not added during immunohistochemistry, were run for each cell type (Fig 6i).

The tissue was rehydrated and blocked with an appropriate blocking solution. Tissue was incubated overnight with primary antibodies (Table 1) followed by incubation with an appropriate fluorescently labeled secondary

antibody (Jackson Immunochemicals, West Grove, PA) at room temperature for 30 minutes.

The lesion site and the surrounding tissue of at least 6 sections per animal per antibody were digitally photographed using a Leica/Spot system (Version 2.2 for Windows, Diagnostic Instruments, Inc. Sterling Heights, MI). The proportional area of tissue occupied by immunohistochemically stained cellular profiles within a defined target area (the lesion site and surrounding tissue) was measured using the Scion Image Analysis system (www.rsb.info.nih.gov/nih-image/) as described previously [Popovich et al., *Supra* (1997)]. All tissue sections were coded prior to measurement to prevent bias and all image backgrounds were normalized prior to quantitation.

Statistical analysis.

Area of spinal cord occupied by cell type is expressed as mean +/- SEM. Kruskal-Wallis statistical analysis with Dunn's post-test was used to compare means (due to large mean number of pixels and large standard errors leading to the necessity of using a non-parametric test). Tests were performed using the Graph Pad Prism Program, Version 3.02 for Windows (GraphPad Software, Inc. San Diego, CA) and SPSS 11.0 for Windows (SPSS, Inc., Chicago, Illinois).

Neutrophils, macrophages/activated microglia and astrocytes were the primary cells found in the lesioned spinal cord. T lymphocytes, B lymphocytes and Schwann cells were also identified. However, based on our measurement of the number of immunopositive pixels in the area surrounding the lesion, there was approximately 80% less ($p<0.0001$) immunolabeling of T and B lymphocytes and Schwann cells than macrophages/activated microglia and astrocytes.

Neutrophils

Immunohistochemical labeling with the antibody against the RP3 clone

revealed small, round, cellular profiles that were detected at all time points investigated in both control and light treated groups (Fig. 6a, b). These cells chiefly bordered the lesion site, but some cellular profiles were perivascular or adjacent to the meninges. The largest amount of positive labeling occurred at 4 dpi. This labeling was significantly increased at this time point for both light treated and control groups when compared to all other time points ($p<0.05$; Fig 6c). However, when the amount of positive immunolabeling for neutrophils in the light treated and control tissues was analyzed, there was no significant difference at any time point (Fig 6c). Immunolabeling for neutrophils decreased after 4 days. At 14 dpi, the remaining neutrophils in the control tissue were found not only along the edges of the lesion but also 1 mm caudal to the lesion edge. Neutrophil migration was not found in any of the light treated tissue.

Macrophages/Activated Microglia

Macrophages and activated microglia are not distinguishable from each other in the mammalian CNS since activated microglia express the same cell surface molecules and cytokines and have the same round morphology as blood borne macrophages [Popovich et al., *J. Comp Neurol* 377:443-464 (1997); Carlson et al., *Exp Neurol* 151:77-88 (1998)]. Immunolabeling for ED1, an antibody against a macrophage/microglia lysosomal glycoprotein revealed many of these large, amoeboid cells in the injured spinal cord (Fig 6d - h). At 48 hours post-injury, immunopositive macrophages/activated microglia were located in and around blood vessels, in the dorsal roots and along the edges of the lesion site, with no infiltration into the surrounding tissue. At this time point, there were observably fewer labeled macrophages/activated microglia in the light treated group than in the control group. By 4 dpi, a large cavity had formed and macrophages/activated microglia were localized to the lesion edges. Similar to the 48 hour situation, there were significantly fewer immunolabeled cells at 4 dpi in

the light treated tissue compared to the control tissue. By 14 dpi, the immuno-positive cells were found along the edges of the lesion and within the lesion cavity (Fig 2d), and had also invaded the tissue rostral and caudal to the lesion site, reaching up to 4 mm rostral to the lesion and 2 – 3 mm caudal. This cellular migration was predominantly in the white matter of the dorsal funiculus around small cavities in the rostral/caudal tissue. The light treatment group had less migration of macrophage/activated microglia at this time point (Fig 6e), with migration reaching approximately 1 mm in the rostral direction and absent in the caudal direction. At 16 dpi, there appeared to be fewer ED1 labeled cells in the control tissue, so that control and light treated tissue looked similar (Fig 6g, h).

In both control and light treated groups, ED1 expression showed an initial peak at 48 hours post-injury and a subsequent peak at 14 dpi, with a decline at 4 dpi. Both peaks were reduced in the light treated group, with significant reductions in ED I expression at 4 and 14 dpi in the light treated group ($p<0.001$, Fig 6f). Moreover, ED1 expression was further reduced at 5 weeks post-injury in the light treated group compared to controls ($p<0.00$ 1, Fig 6f). While there wasn't a significant decrease ($p=0.156$) in ED1 expression in the light treated group at 48 hours post-injury, a trend toward suppression of ED1 expression in the light treated group in comparison to the control group was found.

Astrocytes

Astrocytes were detected using an antibody against GFAP, an intermediate filament primarily expressed in astrocytes. Immunolabeling with this antibody revealed long thin processes that were heavily labeled near the lesion site in both the light treated and control groups. GFAP positive processes were also found throughout the entire length (10 mm) of the sections studied, extending 3 mm rostral and 5 mm caudal to the lesion. At 48 hours post-injury, heavy GFAP

positive labeling was found to outline the lesion in all rats of the control group and GFAP positive processes were found throughout the 10 mm section in 3 of the 5 rats of the control group (Fig 7a). Light treated tissue, however, had only a light band of GFAP positive label near the lesion edge and along the meninges/blood vessels in all 5 rats (Fig 7b). By 4 dpi, however, GFAP labeling in the light treated tissue had increased to the degree observed in the 48 hour control tissue in 3 of the 5 rats. In both groups, immunolabeling for GFAP decreased over the remaining time periods (Fig 7c – f), eventually becoming restricted to the lesion site by 5 weeks post-injury. Interestingly, at 16 dpi, 2 days after light treatment ended, there was a slight increase in rostral/caudal extension of GFAP labeling in 3 of the 5 rats in the light treated group (Fig 7f).

Quantitative analysis revealed that there was a significant decrease in GFAP expression in the light treated group at 48 hours post-injury compared to the control group ($p<0.05$), and a significant increase at 4 dpi compared to the control group (Figure 7g, $p<0.01$). Expression peaked in the control group at 48 hours post-injury, and declined significantly ($p<0.05$) thereafter.

T Lymphocytes

T lymphocytes were detected in spinal cord tissue using UCHL1, an antibody against the surface glycoprotein CD45. Cells that were immuno-positive for UCHL1, were small, round cells and were found in very low numbers. T lymphocytes were restricted to the lesion edge and in the acellular matrix within the lesion cavity (Fig 8a – b).

Statistical analysis of UCHL1 expression revealed that there was a peak in both the control and light treated groups at 48 hours post-injury, with a decline in expression through 16 dpi (Fig 8c). UCHL1 expression in the light treated group was lower than the control group at 48 hours, 14 and 16 dpi, with a significant

decrease found at 14 dpi ($p<0.001$).

B Lymphocytes

B lymphocytes, identified using the L26 antibody against CD20, a membrane spanning protein in B lymphocytes, were also found in very low numbers from 4 to 16 dpi. At the time points studied, L26 expression was found to be in small, round cells near the edges of the spinal cord lesion (Fig 8d) or within the cavity, with 1 – 2 mm migration caudal to the lesion in the white matter tract at 16 dpi in the control group only. There was no migration observed in the light treated group. Quantitative analysis of L26 expression found no significant differences between the light treated and control groups, although a non-significant trend towards a suppression of B lymphocyte activation was observed in the light treated group at 16 dpi (Fig 8e).

Schwann Cells

Also present in very low numbers were Schwann cells, identified by antibody labeling of S100, a neural specific Ca^{2+} binding protein. These small, circular cells were found at all time points investigated, primarily along the edges of the lesion (Fig 8f – g), without any migration rostral or caudal to the lesion. There was no significant difference in expression between LPLI treated and control tissue at any time point (Fig 8h).

Axons have the inherent ability to regrow following injury. Altering the spinal cord environment may support this regeneration. Transcutaneous application of light significantly affected invasion and activation of a number of cells that have a profound influence on recovery after SCI, including macrophages, microglia, astrocytes and T lymphocytes. The results of this study show that LPLI not only caused a significant inhibition of activation and invasion of several cell types, but also shifted the peak activation time in other cell types after SCI. This

alteration in the temporal course of cellular reactions results in a change in the spinal cord environment at a time when axons are sprouting and entering the lesion zone (Fishman and Mattu, 1993). These results serve as a foundation for the novel concept of using transcutaneous application of light to promote axonal regeneration and functional recovery after SCI.

Example 16. RT-PCR analysis of gene expression

At 6 hours or 4 days post-injury, rats (n=5/group/time point) were deeply anesthetized with chloral hydrate (1 ml/100 g, I.P., 10% solution) and euthanised by decapitation. The 5 mm of the spinal cord encompassing the lesion site and the area immediately rostral and caudal to the lesion site were dissected rapidly and placed in 500 µl of RNAlator solution (Ambion, Austin, TX). Total cellular RNA was extracted using the Trizol (Invitrogen, Carlsbad, CA)/phenol (Sigma, St. Louis, MO)/chloroform (Sigma) technique and reverse transcribed using First-Strand Synthesis beads (Amersham Pharmacia, Piscataway, NJ) as per the protocol of the manufacturers (Invitrogen and Amersham Pharmacia). Resultant cDNA was amplified using the CytoXpress Multiplex Inflammatory Set 1 (Biosource, Camarillo, CA) or primers specific for genes of interest (Table 1). Unless otherwise noted, primer sequences were obtained with the use of the Primer3 program (Rozen and Skaletsky, 2000), with complete cDNA sequences obtained from the NIH GeneBank Entrez program. Negative (no sample added to PCR mix) and positive (provided with kit) controls were included in each PCR assay to ensure that contamination was avoided.

PCR products were assessed by electrophoresis on a 2% agarose gel containing ethidium bromide (Sigma). PCR bands were visualized using UV light and photographed. Scion Image (www.rsb.info.nih.gov/nih-image/) was used to measure band pixel density, reflecting relative gene expression. Adjustment was

performed to normalize pixel intensity for samples run on different gels in order to compare the data. Pixel density for each band was obtained and normalized against the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All data is presented as the ratio of the gene of interest to GAPDH.

Resultant relative gene expression is presented as mean ratio +/- SEM. One-way ANOVA was used to compare groups. Tukey's Multiple Comparison test served as a post-test to the ANOVA for comparison of individual groups. All statistical analyses were performed using the GraphPad Prism Program, Version 3.02 for Windows (GraphPad Software, Inc. San Diego, CA).

Comparison of resultant bands to the molecular weight marker confirmed that each investigated gene appeared at the expected molecular weight (data not shown). Analysis of gene expression at 6 hours and 4 days post-injury revealed that all genes were detected at all time points investigated, and no significant difference ($p=0.6740$) was found in expression of GAPDH between the control and LT groups (Fig 9). Expression profiles from each sample were only included if expression for the internal control, GAPDH, could be detected. Gene expression of GAPDH for one sample of the LT-4 day group was insufficient for measurement, so this sample was not included in any data analysis. Therefore, data for this group are from four animals; all other groups were comprised of five rats.

Example 17. Pro-inflammatory Cytokine expression in LPLI treated animals

The expression of four pro-inflammatory cytokines, IL1β, TNFα, IL6 and GM-CSF, was assessed at 6 hours and 4 days post-injury. LPLI immediately following injury resulted in a significant suppression ($p<0.001$; Fig 10a, b) of IL6 expression at 6 hours post-injury. A 171-fold decrease in expression of IL6 in the LT group as compared to the control group was detected at this time point. By 4 days, transcription of IL6 had significantly decreased by 58% in the control group

($p<0.001$, Fig 10a). The expression in the LT group remained depressed from 6 hours to 4 days, but there was no significant difference between IL6 levels at 4 days post-injury between the control and LT groups. There was no significant difference between control and LT groups in expression of TNFα, IL1β and GM-CSF at 6 hours post-injury or 4 days post-injury (Fig 10c - e).However, a trend was found in expression of GM-CSF at both 6 hours and 4 days post-injury, with a 10 and 3 fold decrease in expression found between the LT and control groups, respectively (Fig 10e). A trend toward increase in transcription of TNFα in the LT group at 4 days post-injury was shown, although this increase was not significantly different from the control group (Fig 10d).

Example 18. Chemokines expression in LPLI treated animals

Two chemokine genes were examined in this study. Analysis of mRNA quantities for MIP1α and MCP-1 were performed at 6 hours and 4 days post-injury. Quantitation of transcription revealed that LT resulted in a significant decrease in MCP-1 at 6 hours post-injury ($p<0.01$, Fig 11a, b). The control group at this time point was found to have 66% greater expression of MCP than the LT group. This reduction in expression continued through 4 days post-injury, with a two-fold decrease in MCP-1 expression in the LT group, although this difference between the two groups was not significant. No significant differences between the LT or control group were found at 6 hours or 4 days post-injury for MIP1α (Fig 11c); however there was a five-fold increase in MIP1α expression at 4 days post-injury in the LT group.

Example 19. ICAM, INOS, TGFβ expression in LPLI treated animals

Gene expression was also evaluated for INOS, ICAM and TGFβ. Analysis of gene expression revealed that LT resulted in a five-fold suppression of iNOS transcription at 6 hours post-injury ($p<0.01$; Fig 12a), and a four-fold decrease in

iNOS at 4 days post-injury that did not reach statistical significance. Again, similar to the situation with IL6 and MCP-1, INOS expression was significantly decreased ($p<0.01$, Fig 12a) in the control group from 6 hours to 4 days post-injury, but the expression levels were relatively constant over this time period in the LT group. TGFβ and ICAM, however, demonstrated a trend towards increase at both 6 hours and 4 days after injury in the LT group, although this increase did not reach statistical significance (Fig 12b, c).

The above description is for the purpose of teaching the person of ordinary skill in the art how to practice the present invention, and it is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention, which is defined by the following claims. The claims are intended to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates the contrary.

TABLE 1

Beam Transmission Calculations -- Tests at USUHS 4/02/03

Define:

ω = 1/e² Beam Radius (cm), best guess scattered beam size
r1 = Detector's Aperture, Radius (cm)
r2 = Input Beam Radius (cm)

θ_1 = Integration Limit -- Full Circle

Power Density = 0.750 W/cm²

Assume: Gaussian Intensity distribution

$\omega := 3.55$ $\quad r1 := .05$ $\quad \theta_1 := 2\cdot\pi$ $\quad r2 := 1.5$ $\quad BeamArea := \pi\cdot r2^2$

$e^{-2\left(\frac{\omega}{\omega}\right)^2} = 1.353\times 10^{-1}$ $\quad BEAMPOWER := BeamArea\cdot .750$ $\quad I_0 := \frac{2\cdot BEAMPOWER}{\pi\cdot r2^2}$

SANITY CHECK

$$POWER(r,\theta) := I_0\int_0^{\theta_1}\int_0^{r2} r\cdot e^{-2\left(\frac{r^2}{r2^2}\right)}\,dr\,d\theta \qquad POWER(r2,\theta_1) = 4.584\times 10^{0}$$

Expect the power at the 1/e points to be 86.5% of total; therefore:

$$(POWERCHECK := BEAMPOWER\cdot .865) = (4.586\times 10^{0})$$

SAMPLE POWER, Centrally Located 1 mm Diameter Circular Detector, 100% Transmission

$TRANSBEAMAREA := \pi\cdot\omega^2$ $\quad It := \frac{(2\cdot BEAMPOWER)}{\pi\cdot\omega^2}$

$$SampleOnCenter := It\cdot\int_0^{\theta_1}\int_0^{r1} r\cdot e^{-2\left(\frac{r^2}{\omega^2}\right)}\,dr\,d\theta$$

$SampleOnCenter = 2.103\times 10^{-3}$

$FullSample := \frac{SampleOnCenter}{.865}$ $\quad FullSample = 2.431\times 10^{-3}$

TRANSMISSION CALCULATIONS
Measured Power, Detector at Gaussian Peak (Centered)
1 mm diameter Circular Detector
Skin, Muscle, and Bone Layers Transmission

$SamplePowerMeasurement := 100\times 10^{-9}$

$$CentralPeakTransmission := \frac{SamplePowerMeasurement}{\int_0^{\theta_1}\int_0^{r1} r\cdot e^{-2\left(\frac{r^2}{\omega^2}\right)}\,dr\,d\theta}$$

$CentralPeakTransmission = 1.273\times 10^{-5}$

$TransmittedPower := \frac{\pi\cdot\omega^2\cdot CentralPeakTransmission}{2}$

$TransmittedPower = 2.521\times 10^{-4}$

$Transmission := \frac{TransmittedPower}{\left(\frac{BEAMPOWER}{.865}\right)}$

$Transmission = 4.113\times 10^{-5}$

CHECK

$TransmissionCheck := \frac{SamplePowerMeasurement}{FullSample}$

$TransmissionCheck = 4.113\times 10^{-5}$

TABLE 1 (CONTINUED)

SAMPLE POWER, Off-Center 1 mm Diameter Circular Detector located a ρ, 100% Transmission

At 5.5 mm off Center (Half way Between "peak" Measurments): $\rho := 5.5$

$$(SensorArea := \pi \cdot r1^2) = (7.854 \times 10^{-3})$$

$$\left(\rho_1 := \rho - \frac{\sqrt{SensorArea}}{2}\right) = (5.456 \times 10^{0})$$

$$\left(\phi_1 := \frac{\sqrt{SensorArea}}{\rho}\right) = (1.611 \times 10^{-2})$$

$$(\rho_2 := \rho_1 + \sqrt{SensorArea}) = (5.544 \times 10^{0})$$

$$\left[PowerOffCenter := It \cdot \int_0^{\phi_1} \int_{\rho_1}^{\rho_2} r \cdot e^{-2 \cdot \left(\frac{r^2}{\omega^2}\right)} dr\, d\theta\right] = (1.731 \times 10^{-5})$$

CHECK

$$ExpectedPowerOffCenter := \frac{(PowerOffCenter \cdot Transmission)}{.865}$$

$$ExpectedPowerOffCenter = 8.232 \times 10^{-10}$$

Note, Measured Power was 100 nW versus the 82 nW calculated, the difference is due to the Gaussian Approximation